

## Short Report: Identification of Oropouche *Orthobunyavirus* in the Cerebrospinal Fluid of Three Patients in the Amazonas, Brazil

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**Abstract.** Oropouche fever is the second most frequent arboviral infection in Brazil, surpassed only by dengue. Oropouche virus (OROV) causes large and explosive outbreaks of acute febrile illness in cities and villages in the Amazon and Central-Plateau regions. Cerebrospinal fluid (CSF) samples from 110 meningoencephalitis patients were analyzed. The RNA extracted from fluid was submitted to reverse transcription-polymerase chain reaction and sequencing to identify OROV. Three CSF samples showed the presence of OROV causing infection in the central nervous system (CNS). These patients are adults. Two of the patients had other diseases affecting CNS and immune systems: neurocysticercosis and acquired immunodeficiency syndrome, respectively. Nucleotide sequence analysis showed that the OROV from the CSF of these patients belonged to genotype I. We show here that severe Oropouche disease is occurring during outbreaks of this virus in Brazil

### INTRODUCTION

Oropouche virus (OROV) is an *Orthobunyavirus* in the *Bunyaviridae* family. These viruses are negative polarity tri-segmented single-stranded RNA viruses. The RNA segments, known as large (L), medium (M), and small (S), encode an RNA-dependent RNA polymerase, envelope surface glycoproteins (Gn and Gc), and a nucleocapsid protein, respectively. The virus replicates in the cytoplasm, buds into the Golgi apparatus, and is excreted by the cell.<sup>1</sup>

Oropouche virus is an arbovirus, transmitted to sloths, marsupials, primates, and birds by *Aedes serratus* and *Culex quinquefasciatus* mosquitoes. Notably, this virus has adapted to an urban cycle involving man, with midges (*Culicoides paraensis*) as the primary vector.<sup>2</sup> Oropouche fever is the second most frequent arboviral infection in Brazil, surpassed only by dengue. Oropouche virus causes large and explosive outbreaks of acute febrile illness in cities and villages in the Amazon and Central-Plateau regions of Brazil. An estimated 500,000 cases of OROV infection have occurred in Brazil in the past 48 years. In addition to outbreaks, OROV can also cause sporadic human infections.<sup>3</sup> We have recently reported an outbreak of OROV, occurring from 2007 through 2008, in Manaus, the capital of Amazonas State, a large city with ~2 million inhabitants.<sup>4</sup> In that report, 128 OROV serologically confirmed patients, 2–81 years of age, were identified. In addition to fever, the patients had headache (72.7%), myalgias (70.3%), and arthralgias (57.8%). Rash was observed in 42.2%, and hemorrhagic phenomena (petechiae, epistaxis, and gingival bleeding) were observed in 15.5%. All patients recovered without sequelae and were not hospitalized. Despite reference of sporadic cases of symptoms of involvement of the central nervous system (CNS) in OROV outbreaks, data concerning CNS symptoms were not given.<sup>4,5</sup>

In Brazil, many CNS infections seemed to be caused by viruses such as enterovirus, cytomegalovirus, herpes simplex virus type 1, varicella-zoster virus or Epstein-Barr virus.<sup>6</sup> In addition to OROV, another *Orthobunyavirus*, the Tucunduba virus has been reported to cause meningoencephalitis in Brazil.<sup>7</sup> However, identification of the virus causing CNS infection is uncommon. We report here three cases of CNS infection by OROV diagnosed using reverse transcription (RT) followed by polymerase chain reaction (PCR) and nucleotide sequencing from RNA extracted from cerebrospinal fluid (CSF).

### MATERIALS AND METHODS

Cerebrospinal fluid samples from 110 meningoencephalitis patients were analyzed. All study patients provided an informed consent and authorized in a signed document divulgation of results obtained as part of this work. This study was approved (nr.0048.0.114.000-07) by the Ethics Review Board of the Tropical Medicine Foundation of Amazonas (FMTAM), Manaus, Amazonas, Brazil.

These patients were hospitalized and treated, from 2005 to 2010, in the Hospital of the Tropical Medicine Foundation of Amazonas (FMTAM), a tertiary care center specializing in tropical and infectious diseases located in the city of Manaus. The analysis of viruses in the CSFs attended to the best interest of the patients as part of the routine diagnostic procedures.

**Detection of virus genomes.** Nucleic acids were extracted from the CSF of the patients by using the QIAmp viral RNA Mini Kit (QIAGEN, Valencia, CA), following the manufacturer's specifications. For the RT-PCR, the reverse transcription was conducted in 5 µL of the RNA extracts with random primers (AccessQuick RT-PCR System, Promega, Madison, WI). The RT-PCR was followed by a nested-PCR, with specific Simbu serogroup primers selected on the basis of the nucleotide sequence of OROV TrVL9760 strain S segment, producing 300 base pair amplicons, as previously reported.<sup>8</sup> The samples were also tested by PCR for herpesvirus (herpes simplex virus types 1 and 2, varicella zoster virus, cytomegalovirus, and Epstein-Barr virus),<sup>9</sup> and by RT-PCR for enterovirus.<sup>10</sup>

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The amplicons were directly sequenced after purification. Aliquots of 40 µL of each amplicon were purified with the QIAmp PCR purification kit (QIAGEN) and sequenced in both directions by using the Simbu serogroup primers and the BigDye Terminator Cycle Sequence Kit v3.1 in an ABI3130x1 automated sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequences were subjected to the basic local alignment search tool (BLAST) ([www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast)) analysis using the megablast algorithm for highly similar sequences.<sup>11</sup>

**Detection of IgM antibodies to OROV.** Immunoglobulin M (IgM) and IgG antibodies to OROV were analyzed in the CSF of the patients by an in-house enzyme immunoassay that uses virus-infected cultured cells as antigen (EIA-ICC).<sup>12</sup>

## RESULTS

Detection of virus genomes was performed in CSF samples by PCR for herpesvirus, RT-PCR for enterovirus, and RT-nested-PCR for OROV. The CSF samples having OROV genome detected by RT-PCR were also tested for IgM- and IgG-specific antibodies to OROV in the CSF of the patients by an in-house EIA.

All of the 110 CSFs samples were negative in the RT-PCR for enterovirus and in the PCR for herpesvirus. Three patients (2.7%) were positive for OROV by RT-nested-PCR. The CSF of the three patients positive for OROV was searched for antibodies to OROV by the EIA-ICC assay. All three patients showed IgG antibodies to OROV in the CSF and one of these patients (patient 2) showed also specific IgM to OROV. These three patients had fever and symptoms of CNS involvement suggesting meningoencephalitis. Cerebrospinal fluid of these patients showed a predominantly lympho-monocytic pattern, suggestive of viral infection, as shown in Table 1. The amplicons obtained from CSFs of the three patients by RT-nested-PCR for OROV were directly sequenced. All three sequences AMLq13 (Gene Bank accession HM107840), AMLq14 (HM107841), and AMLq16 (HM107842), showed 100%, 99%, and 100% similarity, respectively, to the provisional reference sequence of OROV TRVL9760 (AF64531) and to the strain BeAn19991 (AF164532), isolated in Brazil 50 years ago.<sup>13</sup>

To genotype the three OROV detected in this study, their sequences were aligned with a dataset of Oropouche sequences TRVL9760 (AF164531), BeH505663 (AF164543),

BeH505442 (AF164542), DEI209 (AF164551), BeH544552 (AF164546), BeAn19991 (AF164532), BeH475248 (AF164540), GML444477 (AF164555), GML445252 (AF164557), and GML444911 (AF164556) and Aino virus (M22011) as an outgroup, using the ClustalX software.<sup>14</sup> Phylogenetic analysis was conducted with the neighbor-joining method and the Kimura 2-parameter nucleotide substitution model using MEGA 4 software (Figure 1).<sup>15</sup>

## DISCUSSION

Oropouche virus, present only in South America, is one of the most important arbovirus that infects humans in the Brazilian Amazon. In this study, OROV was found to cause CNS infection in three patients. Clinical presentation of OROV infection was first reported by Pinheiro and others<sup>2</sup> and since then, < 10 sporadic cases have been reported. In this study, the three patients with meningoencephalitis presented a clear lympho-monocytic cellular pattern in CSF, high protein, and normal to slightly decreased glucose levels. This is strongly suggestive of a viral infection. Of note, two of these patients presented with underlying infections that may affect the CNS and immune systems. One patient had neurocysticercosis whose diagnosis was based on magnetic resonance suggestive image and the other patient had human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) and herpes zoster. As not all patients infected with neurocysticercosis and HIV/AIDS and herpes zoster developed meningitis, it is probable that the two patients co-infected with OROV progressed to meningitis because of being immunocompromised. The third patient developed meningitis probably because of OROV as no other infections were observed. This infection was also confirmed by the presence of IgM- and IgG-specific antibodies to OROV in the CSF. Thus, it is possible that the invasion of CNS by OROV has been facilitated by previous blood-brain barrier damage. All three patients survived despite a long hospitalization.

Saeed<sup>13</sup> and others<sup>8</sup> reported the first molecular epidemiology analysis of OROV, suggesting the existence of at least three genotypes (I, II, and III) of the virus in the Americas. In this study, OROV from the three patients belonged to genotype I, grouping with the first isolated OROV, from Trinidad TRVL9760, 1955 (AF64531), and the first Brazilian isolate of OROV BeAn19991, 1960 (AF164532). In Brazil, the genotype I was originally isolated in Santa Maria County,

TABLE 1  
Clinical and laboratory data of the 3 patients having infection by OROV in CSF\*

Patient	1	2	3
OROV sequence	AMLq13	AMLq14	AMLq16
Genera/age (years)	Male/54	Male/20	Female/37
Profession/county of origin/year	Fisherman/Uricurituba/September 2006	Agriculture worker/Manacapuru/February 2007	Domestic worker/ Manaus/June 2007
Signs and symptoms	Headache, dizziness, cloud vision, and Romberg sign	Headache, fever, chills and malaise	Headache, nausea, vomiting, and paraplegia
Associated diseases	Neurocysticercosis (diagnosed by a magnetic resonance image)	No reference	HIV/AIDS Herpes zoster
CSF analysis	6 monocytes/mL, glucose: 59 mg/dL, protein: 40 mg/dL	134 cells/mL (100% lympho-monocytic cells), glucose: 50 mg/dL, protein: 107 mg/dL	533 cells/mL (100% lymphomonocytic cells), glucose: 107 mg/dL, protein: 136 mg/dL
Outcome	Survived after 4 days hospitalization	Survived after 24 days hospitalization	Survived after 32 days hospitalization

\*OROV = oropouche virus; CSF = cerebrospinal fluid; HIV = human immunodeficiency virus; AIDS = acquired immunodeficiency syndrome.

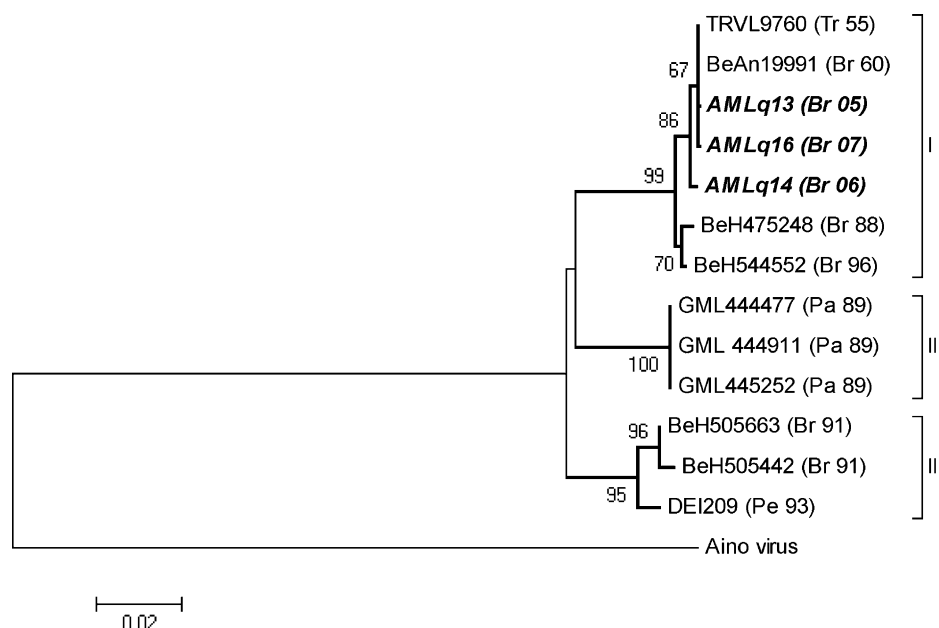


FIGURE 1. Phylogenetic analysis showing Oropouche virus (OROV) genotypes detected by reverse transcription-polymerase chain reaction (RT-PCR) in cerebrospinal fluids of three patients having meningoencephalitis (bold-italics). This neighbor-joining consensus tree was inferred from 1,000 bootstrap replicates. The percentage of replicate trees, which clustered together in the bootstrap testing, is shown next to the branches. Kimura 2-parameter nucleotide substitution model was chosen as the best-fit after a run on FindModel using all 28 models implemented. (<http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html>). Country and year of isolation is referred between brackets.

at the State of Para, and in the following years, it has been reported in outbreaks of acute febrile illness in the State of Amazonas and other western Brazilian States.<sup>3,16</sup> Therefore, the high similarity of the short nucleotide sequences of the conserved *N* gene of OROV, with old OROV isolates reported in this study, could be similar or bears little difference if other parts of the viral genome were analyzed.

Though we only identified three patients here, the occurrence of CNS involvement in OROV infections may be vastly underestimated, especially in patients immunocompromised and those with previous blood-brain barrier disruption. Finally, these cases of severe Oropouche disease show that OROV should be investigated in cases of meningoencephalitis of unknown etiology.

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